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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.53(c).

Docket Number	00786/434001	Type a plus sign (+) inside this box —>	+

INVENTOR(S)/APPLICANT(S)

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TITLE OF THE INVENTION (280 characters max)

COMPOSITIONS, SOLUTIONS, AND METHODS USED FOR TRANSPLANTATION

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ENCLOSED APPLICATION PARTS (check all that apply)

⊠ Specification	Number of pages: 36	⊠ Cover Sheet:	Number of pages: 1 page
⊠ Drawings	Number of sheets: 11	⊠ Claims	Number of pages: 10
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⊠ A check or money order is enclosed to cover the filing fees. ⊠ The Commissioner is hereby authorized to charge filing fees not covered and apply any credits to Deposit Account Number: 03-2095	FILING FEE AMOUNT	$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

⊠ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are:

⊠ Applicant claims small entity status under 37 C.F.R. § 1.27.

Respectfully submitted,

SIGNATURE: [signature] DATE: 18 October 2002

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PROVISIONAL APPLICATION

UNDER 37 C.F.R. § 1.53(c)

APPLICANT : FRANÇOIS BERTHIAUME, MARTIN YARMUSH, AND YASUJI MOKUNO

TITLE: COMPOSITIONS, SOLUTIONS, AND METHODS USED FOR TRANSPLANTATION

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PATENT
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COMPOSITIONS, SOLUTIONS, AND METHODS USED FOR TRANSPLANTATION

Statement as to Federally Sponsored Research

This invention was funded, in part, by grants from the National Institute of Health. The government may have certain rights in the invention.

Background of the Invention

The invention relates to cell, tissue, and organ transplantation.

Currently, the major impediment limiting clinical transplantation is the persistent shortage of organs which often results in extensive numbers of patients on transplant wait-lists as well as patients dying before a suitable transplant can be found. In the context of liver transplantation, although the majority of liver donors are cadaveric, living and split liver donor techniques are promising alternatives, yet represent only about 3% of the total number of transplants performed in the United States (Sindhi *et al.*, *J. Ped. Surg.* 34: 107-110, 1999). Furthermore, living donor methods are inherently limited because they represent a significant risk for the donor. Another approach is the use of bioartificial liver support systems which may provide temporary liver function support and, in cases where the patient could recover from the acute phase of the disease, avoid the need for a liver transplant altogether. However, given that such systems are still in very early stages of development, cadaveric organs are likely to remain the main donor source in the foreseeable future, and this source is unlikely to rise in the face of enhanced safety measures, which prevent accidents.

As such, about two thousand potential liver recipients on transplant wait

lists die every year as a consequence of the shortage in organs. Furthermore, a significant number of donor organs are discarded and eliminated from the donor pool even before transplantation, thus exacerbating the problem of organ shortage. In the context of liver transplants, a significant proportion of donor livers is steatotic or fatty and thus often considered to be unacceptable for transplantation purposes. Although usually asymptomatic, the accumulation of lipid in livers, also known as hepatic steatosis, is the most common single predisposing risk factor for postoperative liver failure and accordingly, approximately 65% of livers rejected for transplantation are steatotic (Urena *et al., World J. Surg.* 22: 837-844, 1998). In fact, it is noteworthy that no single other liver pathology is as prevalent as steatosis and is associated with such a negative impact on the current shortage of donor livers.

Indeed, data from animal models suggest that steatotic livers are far more susceptible to ischemia-reperfusion (I/R) related damage than so-called lean livers. In this respect, I/R causes necrosis and apoptosis of hepatocytes and endothelial cells through the generation of oxygen reactive species and the disruption of the microvasculature, ultimately leading to hepatic failure. Studies on the effect of cold storage of liver followed by rewarming and perfusion also show more extensive damage in fatty livers and a reduced "safe" preservation time before transplantation. In the context of liver transplantation, lipid accumulation in the liver also impairs certain key liver functions namely glucose production and cytochrome p450 detoxification activity (Gupta *et al.*, *Am. J. Physiol.* 278:E985-E991, 2000; Leclercq *et al.*, *Biochem. Biophys. Res. Commun.* 268: 337-344, 2000).

Furthermore, livers with mild to moderate steatosis, which are even

considered marginally acceptable, have a lower graft survival rate (76% vs. 89% for lean livers) at 4 months post-transplantation. Patients receiving steatotic livers have a 77% survival rate at two years post-transplantation compared to a 91% survival in patients receiving nonsteatotic livers. It is clear that methods, which would salvage or recondition donor livers discarded because of severe steatosis or increase the success rate of transplanted steatotic livers would significantly reduce the number of patient deaths, and help to bridge the gap existing between supply and demand in liver transplantation.

Summary of the Invention

As is described in greater detail herein, this invention provides a method to metabolically eliminate intracellular lipid storage in liver tissue. It is useful because it provides for an efficient means to rapidly remove excess lipid storage from virtually any potential source of donor material (such as a cell, tissue, or organ) which are deemed unacceptable for transplantation due to their high fat content. This method has important applications to transplantation because it will significantly increase the pool size of available donor material and, as a result, alleviate the current severe shortage of such material, including donor livers. This, in turn, translates into a reduction in the number of patients on the liver transplant waiting list and the number of patients dying before a suitable transplant is found.

In one aspect, the invention features a method for preparing a donor cell, tissue, or organ for transplantation into a recipient, where the method includes reducing intracellular lipid storage material of the cell, tissue, or organ. In preferred embodiments, a human liver cell; human liver tissue; or a human liver organ is prepared.

Preferably, the method of reducing intracellular lipid storage material includes contacting the cell, tissue, or organ with a solution (such as the defatting solution described herein) that increases oxidation of a lipid; export of a lipid from the cell, tissue, or organ; or that increases oxidation and export of a lipid from the cell, tissue, or organ. In preferred embodiments, the method results in reducing an ischemia-reperfusion injury in the cell, tissue, or organ upon transplantation into a recipient or results in reducing a cold-preservation-related injury in the cell, tissue, or organ upon transplantation into a recipient. In other preferred embodiments, the method reconditions a steatotic cell, tissue, or organ.

In another aspect, the invention features a solution (e.g., a defatting solution) for reducing intracellular lipid storage material of a donor cell, tissue, or organ for transplantation into a recipient, including a catabolic hormone, and an amino acid. In preferred embodiments, the catabolic hormone of the solution increases intracellular lipid oxidation; lipid export; or both. Exemplary catabolic hormones include glucagon, epinephrine, growth hormone, hepatocyte growth factor, leptin, thyroid hormone, or a glucocorticoid hormone (such as a hydrocortisone, a cortisol, a corticosterone, or dexamethasone). In still other preferred embodiments, an amino acid (such as alanine or glutamine) is required for the synthesis of an apolipoprotein. In yet other preferred embodiments, the solution further includes an anti-oxidant or an oxygen carrier. Exemplary anti-oxidants include N-acetyl-cysteine, glutathione, allopurinol, or adenosine, and exemplary oxygen carriers include hemoglobin or a perfluorocarbon. If desired, the solution optionally includes a component that provides oncotic pressure.

In preferred embodiments, the solution includes: from 50 mM to 150 mM sodium ion; from 0.4 mM to 4 mM potassium ion; from 0 mM to 50 mM

phosphate ion; from 0 mM to 44 mM bicarbonate ion; from 0.19 mM to 5 mM calcium ion; from 0.081 mM to 5 mM magnesium ion; from 0.2 mM to 2.4 mM alanine; from 0.2 mM to 10 mM glutamine; from 50 pg/mL to 1000 pg/mL glucagon; from 100 pg/mL to 2500 pg/mL epinephrine; from 50 ng/mL to 1500 ng/mL hydrocortisone; and from 30 g/mL to 120 g/mL hydroxyethyl starch.

In still other preferred embodiments, the solution includes: 116 mM sodium ion; 2.3 mM potassium ion; 1.0 mM sodium phosphate (monobasic); 26 mM sodium bicarbonate; 1.9 mM calcium ion; 0.81 mM magnesium ion; 0.48 mM alanine; 2.00 mM glutamine; 100 pg/mL glucagon; 250 pg/mL epinephrine; 150 ng/mL hydrocortisone; and 60.0 g/mL hydroxyethyl starch.

Preferably, the solution is heated to a temperature of 25 to 40 °C, such as to 37 °C; is exposed to 20 to 100% O_2, such as 95% O_2; is exposed to 0 to 10% CO_2, such as 5% CO_2; and has a pH of 6.5 to 7.8, such as a pH of 7.4.

In still another aspect, the invention features a method for preparing a donor cell, tissue, or organ (including steatotic cells, tissues, or organs) for transplantation into a recipient, the method includes contacting the donor cell, tissue, or organ with any of the aforementioned solutions.

Additionally, the invention features a method of storing or preserving a donor cell, tissue, or organ for transplantation into a recipient, the method includes contacting the donor cell, tissue, or organ with any of the aforementioned solutions.

The invention further features kits for preparing or storing a donor cell, tissue, or organ for transplantation into a recipient (including kits for reconditioning steatotic cells, tissues, or organs), the kit including a solution for reducing intracellular lipid storage material of the donor cell, tissue, or organ and

instructions for using the solution(s) provided in the kit.

In another aspect, the invention features a cell, tissue, or organ prepared according to any one of the aforementioned methods involving the reduction of intracellular lipid storage material, and therefore includes isolated defatted donor cells, tissues, or organs that may be used for transplantation into a recipient.

In a final aspect, the invention features a method of transplanting a cell, tissue, or organ, the method including (a) providing any of the aforementioned defatted cells, tissues, or organs; and (b) transplanting such a cell, tissue, or organ into a recipient.

By "lipid storage material" is meant any of a variety of cellular substances that are soluble in nonpolar organic solvents. Such material includes, without limitation, triglycerides, cholesterol, cholesterol esters, free fatty acids, and phospholipids.

By "reducing intracellular lipid storage material" is meant decreasing an amount of lipid storage material in a cell, tissue, or organ by inducing catabolic metabolism of the lipid storage material by increasing lipid export, lipid oxidation, or both from the cell, tissue, or organ. Typically, the intracellular lipid storage material of a donor cell, tissue, or organ is measured relative to the intracellular lipid storage content a control cell, tissue, or organ. In preferred embodiments, the lipid storage material of a donor cell, tissue, or organ is reduced by at least 20% (and preferably 30% or 40%) as compared to the lipid storage material of a control cell, tissue, or organ. In other preferred embodiments, the lipid storage material is reduced by at least 50%, 60%, and more preferably reduced by 75%, 80%, 85%, or even 90% of the level of a control; with up to a 95% reduction in lipid storage material as compared to a control being most preferred. The level of lipid storage

material is measured using conventional methods, such as those described herein. A reduction in the intracellular lipid storage material of a cell, tissue, or organ is referred to as defatting.

By "ischemia-reperfusion related injury" is meant any damage, including loss of viability, caused to a donor cell, tissue, or organ subsequent to a decrease in the availability of oxygen followed by a sudden increase in oxygen levels. Ischemic or hypoxic conditions for the purposes of the present invention are typically caused by (1) surgical procedures, which require temporary blood flow arrest, including for example liver resection and vascular reconstruction, and (2) storage of the cell, tissue, or organ in the absence of a continuous supply of oxygen. Such conditions allow for the generation of inflammatory mediators, reactive oxygen species, and nitric oxide, as well as the infiltration of neutrophils, which can severely damage cells, tissues, and organs. The length of time necessary for ischemia-related damage is tissue-dependent and certain cells, tissues, or organs may be more susceptible to hypoxic donations as a result of their high-energy demands.

By "cold-preservation related injury" is meant any damage caused to the cell, tissue, or organ caused by the storage of a cell, tissue, or organ in hypothermic conditions for transplantation purposes. As an example, under hypothermic conditions, phospholipids forming the lipid bilayer of the cellular membranes undergo a phase change leading to a reduction in fluidity. As a result of this phase change, the cell fails to utilize oxygen as efficiently, in a situation analogous to anoxic conditions.

By "anti-oxidant" is meant any agent that scavenges reactive oxygen species, which are generated in instances in which oxygen tension is increased.

Changes in oxygen tension may result from a transition from anoxic to normoxic conditions, or from normoxic to supraphysiological oxygen tension. Examples of anti-oxidants include but are not limited to N-acetyl-cysteine, glutathione, allopurinol, adenosine, cyclodextrin, superoxide dismutase (SOD), catalase, chlorpromazine, and prostacyclin.

By "reconditioning a cell, tissue, or organ for transplantation" is meant restoring a cell, tissue, or organ, which is deemed unacceptable for transplantation, into a transplantable form.

The invention is advantageous for several reasons: (1) it will increase the donor pool size, as severely steatotic organs (e.g., livers) are usually discarded; (2) it will improve the outcome of patients who receive organ transplants with mild to moderate steatosis; (3) it will provide the scientific and technology bases to develop similar approaches in a variety of organ systems prone to steatosis during obesity, such as pancreatic β cells and cardiomyocytes; (4) it will provide methods for preventing or limiting hepatic fibrosis, as hepatic steatosis often precedes fibrosis in degenerative liver diseases; and (5) it will further optimize organ preservation techniques and exploit the potential of long-term warm perfusion preservation techniques. Furthermore, the metabolic pre-conditioning regimens of the invention that reduce the lipid load and modulate the redox state of cells (e.g., liver cells) will reduce the impact of I/R and prolong the preservation time of donor livers.

Other features and advantages of the invention will be apparent from the following Detailed Description, the drawings, and the claims.

Brief Description of the Drawings

Figure 1 shows a schematic diagram of a perfusion apparatus used to defat livers. The liver is immersed in the perfusate solution and perfused via the portal vein at a rate of 4 mL/min/g liver. The perfusate is heated to 37^{0}C through a heat exchanger and oxygenated by passing through a thin silicone tubular membrane exposed to 95% oxygen and 5% carbon dioxide. A bubble trap is placed immediately before the perfusate enters the liver.

Figure 2 shows the morphological appearance of cultured hepatocytes after 7 days of plasma exposure (panels A-D). Panel E shows a graph of the intracellular triglyceride levels in hepatocytes for conditions shown in panels A-D. Statistical differences were determined using ANOVA with Tukey's post hoc test (n = 11).

Figure 3 shows the effect of defatting medium on cultured hepatocyte appearance after 2 days of defatting.

Figure 4 shows the release of lactate dehydrogenase by cultured hepatocytes after I/R at 37^{0}C. The left panel shows the effect of hypoxic time before reoxygenation in steatotic and normal "lean" hepatocytes. The right panel shows the effect of defatting time on the response of steatotic hepatocytes to I/R.

Figure 5 shows the release of lactate dehydrogenase (LDH) by cultured hepatocytes in response to 12 hours of storage at 4^{0}C followed by rewarming at 37^{0}C. The left panel shows LDH activity after 12 hours in University of Wisconsin (UW) solution at 4^{0}C and 12 more hours at 37^{0}C in medium. The right panel shows the effect of defatting time on the response of steatotic hepatocytes to cold storage followed by rewarming.

Figure 6 shows the proportion of cytochrome c detected in the cytosolic

fraction of hepatocytes after 12 hours of storage at 4^0C followed by rewarming at 37^0C. Cytosolic cytochrome c is normalized to total (cytosolic + mitochondrial) cytochrome c.

Figure 7 shows the effect of hepatocyte island size and steatosis on hepatocyte viability after I/R. The left panel shows intensity of calcein fluorescence per surface area over hepatocyte islands at various time points during I/R of steatotic hepatocytes co-cultured with nonparenchymal cells. The right panel shows calcein fluorescence per surface area over hepatocyte islands at the 4 hour time point (1 hour of no flow followed by 3 hours of flow).

Figure 8 shows the aliphatic proton signal intensity from proton chemical shift imaging as a function of liver triglyceride content in fatty livers of rats. The correlation coefficient of the linear fit is 0.97.

Figure 9 shows that rats fed a choline and methionine-deficient diet (CMDD) developed fatty livers. Panel A shows the kinetics of hepatic triglyceride (TG) accumulation in rats fed a CMDD for up to 6 weeks. Panel B shows the restoration of the hepatic TG content to normal levels upon return of CMDD animals to a regular diet.

Figure 10 shows that defatting makes fatty donor livers suitable for transplantation. Survival curves for rats receiving donor livers are shown. Livers were stored for 6 hours in UW solution prior to transplantation. CMDD refers to fatty liver recipients. CMDD+RF 3d or 7d refers to recipients receiving donor livers from CMDD fed rats followed by refeeding (RF) with a normal diet for 3 or 7 days, respectively.

Figure 11 shows the effect of amino acids in the perfusate on liver triglyceride content after 3 hours of warm perfusion (panel A) and the effect of

perfusion time on liver triglyceride content using amino acid-containing perfusate (panel B). Fatty livers from CMDD fed rats for 6 weeks were perfused at 37^{0}C. After a 3 hour perfusion, the remaining TG content is in the normal range (~10 mg/g liver).

Detailed Description

The invention provides methods and solutions for metabolic preconditioning of a donor cell, tissue, or organ that reduce intracellular lipid storage materials of such cells, tissues, or organs. The invention further increases the ability of a donor cell, tissue, or organ to withstand ischemia/reperfusion injury (I/R), a cold-preservation injury, or both. The metabolic conditioning methods described herein further improve the outcome of virtually any transplant surgical procedures and reduce the risk of postoperative organ dysfunction to a level similar to that observed in nonsteatotic organs (e.g., livers).

Metabolic Conditioning

Based on our identification of critical branch points of the hepatic metabolic network affected by lipid loading, we provide methods and solutions useful for reducing lipid storage in donor cells, tissues, or organs. To this end, two strategies may be used to reduce the lipid load, these include: (1) hormonal modulation and (2) amino acid supplementation. With the ultimate goal of using lipid lowering techniques in the clinic, noninvasive methods for monitoring the "delipidization" process may also be employed in the methods of the invention. Exemplary monitoring methods include NMR and PET for quantitatively assessing lipid load and metabolism; furthermore, a judicious choice of probes can be used to

simultaneously monitor the quality of perfusion and the energy status of the cells, tissues, or organs.

Optimization of a Metabolic Network

By way of example, lipids are stored in the liver mainly as triglycerides, which can be removed by catabolic action, where one molecule of triglyceride is broken down into one molecule of glycerol and three molecules of fatty acids, followed by β-oxidation of the fatty acids in the mitochondria to generate reducing equivalents, CO_2, and ketone bodies. Triglycerides can also be removed by export in the form of lipoproteins. Thus, the methods of the invention involve maximizing the sum of fluxes represented by β-oxidation and triglyceride export. Furthermore, the methods involve maintaining the intracellular triglyceride synthesis flux to a minimum. These three fluxes are related to each other as well as to the other metabolic fluxes via the stoichiometry of the hepatic metabolic network, which imposes mass balance constraints to the set of possible fluxes.

Experimental Optimization of Fluxes

The predicted optimum fluxes will be induced experimentally by a combination of mass action effects, for example, by altering amino acid levels in the perfusate or culture medium, and hormones which favor fatty acid oxidation and export of triglycerides, e.g., glucagon, epinephrine, growth hormone, hepatocyte growth factor, thyroid hormone, leptin and various glucocorticoid hormones. The steatotic hepatocyte culture system described herein is used in this optimization effort, and the most effective regimen is then utilized in the steatotic perfused liver system. Results from the first studies are analyzed and re-fed into a

linear optimization routine in order to generate other predicted optimum perfusate compositions, which are then utilized for treating a donor cell, tissue, or organ. Going through several iterations with this process, the levels of all components of the perfusate may be optimized. Other optimization methods, such as those using empirical simplex algorithms may be used as well.

During the experiments, culture medium/perfusate samples are obtained at regular intervals and the intrahepatic content of triglycerides and glycogen determined as well. Cultured hepatocyte defatting experiments are performed for 24-48 hours and liver perfusions up to 3 hours, which is sufficient to assess the effect of the defatting procedure. Control hepatocytes or livers from littermates are not defatted and instead used to provide the initial values of lipid/glycogen content. Throughout these studies, metabolic flux analyses are performed to characterize the lipid lowering mechanisms, and determine whether the cellular metabolic state returns to that found in normal nonsteatotic livers as the lipid load disappears. To help in the optimization aspects of defatting perfused livers, noninvasive fat measurement methods based on proton chemical shift NMR imaging and PET using 1-[^{11}C]-3-R,S-methylheptadecanoic acid as a tracer are used to follow the process of delipidization in real time.

Defatting Solutions

Organ preservation and perfusate solutions are known in the art as comprising a base solution that consists of a buffered physiological solution, such as a salt solution or a cell culture-like basal medium, to which is added a variety of defined supplements. In a preferred embodiment, the defatting solution of the present invention also employs such a base solution containing amino acids, ions

(e.g., sodium ion, potassium ion, phosphate ion, calcium ion, magnesium ion, and bicarbonate ion), physiologic salts, impermeants, serum proteins and/or factors, and sugars (e.g., glucose). In addition to the components of the base solution, the defatting solution of the present invention contains a novel combination of supplements that can be grouped in at least 2 component categories. It can be appreciated by those skilled in the art that the components in each category may be substituted with a functionally equivalent compound to achieve the same result. Thus, the following listed species of components in each component category is for purposes of illustration, and not limitation.

A first component category, hormones, comprises a combination of components in a physiologically effective amount, which provide a means to reduce the lipid content in a cell, tissue, or organ by increasing lipid oxidation and lipid export from the cell, tissue, or organ. To insure that this catabolic activity in the cell, tissue, or organ is maintained, conditions characteristic of starvation and thus amenable to lipid reduction are provided. These conditions may include high concentrations of catabolic hormones (e.g., glucagon, epinephrine, growth hormone, hepatocyte growth factor, thyroid hormone, leptin, or glucocorticoid hormones including for example hydrocortisone, corticosterone, cortisol and dexamethasone) and low concentrations of anabolic hormones (e.g., insulin). The result of using such a combination of hormones simulate conditions of starvation in a mammal and as such, the lipid content of a cell, tissue or organ is effectively reduced through the oxidation and the export of lipids. The hormones comprise from about 1×10^{-6} % to about 3×10^{-5} % by volume (w/v) of the novel combination of supplements, which are added to the base solution in forming the defatting solution of the present invention.

A second component category, amino acids, comprises a combination of components in a physiologically effective amount, which provide a means to supply the building blocks required for the synthesis of apolipoproteins, which are subsequently incorporated into larger lipoproteins. These lipoproteins export triglycerides and other lipids (e.g., cholesterol, cholesterol esters, and phopholipids) outside of the cell, tissue, or organ. Such amino acids are added to the defatting solution may include any of the essential nutritional amino acid such as alanine, arginine, aspargine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine; and a combination thereof. The amino acids comprise from about 0.01 % to about 1 % by volume of the novel combination of supplements, which are added to the base solution in forming the defatting solution of the present invention.

It will be appreciated by those skilled in the art that components in any one or more of the two component categories can have additional functions desirable for the process according to the present invention. For example, amino acids contained in the defatting solution include cysteine in amounts which, besides functioning as a building block for lipoproteins, also functions as an antioxidant-preferred free radical scavengers which scavenge toxic free radicals during the flushing and perfusing steps of the process. These toxic free radicals are generated in instances in which oxygen tension is increased (e.g., transition from anoxic to normoxic conditions, or from normoxic to supraphysiological oxygen tension). Other antioxidants, including for example N-acetyl-cysteine, glutathione, allopurinol, adenosine, cyclodextrin, superoxide dismutase (SOD), catalase, chlorpromazine, and prostacyclin may be included, or used as functionally

equivalent compounds, in the defatting solution of the present invention. Such antioxidants comprise from about 0.00 % to about 5.00 % by volume of the novel combination of supplements, which are added to, and dissolved in, the base solution in forming the defatting solution of the present invention.

In another embodiment of the present invention, the defatting solution may further comprise cytoprotective agents, which can prevent apoptosis of cells resulting from the production of ceramide, a bi-product of lipid accumulation. Such cytoprotective agents can include, for example, membrane-permeable peptidic caspase inhibitors, cyclosporin A, and the inhibitor of ceramide production L-cycloserine. Other agents such as vitamins (e.g., choline chloride, folic acid, myo-inositol, niacinamide, pantothenic acid, pyridoxal HCl, riboflavin, thiamine HCL), ions (e.g., sodium chloride, potassium sulfate, sodium phosphate (monobasic), sodium bicarbonate, calcium chloride, and magnesium sulfate), carbohydrates (e.g., glucose) and pH indicators (e.g., phenol red) may also be included in the defatting solution. Optionally, the defatting solution may also contain agents, which can decrease lipid peroxidation, neutrophil infiltration, microcirculatory alterations, and the release of proinflammatory mediators such as TNF-α. The addition of such agents would provide a means to minimize any damage caused by ischemia-reperfusion injury. Agents which can provide oncotic pressure may also be supplemented to the defatting solution and can include but are not limited to albumin, hydroxyethyl starch, or any high molecular weight polymer.

In another embodiment of the present invention, to avoid the use of supraphysiological oxygen tension and perfusion flow rate, the defatting solution contains one or more oxygen transporting compounds ("oxygen carrying agents")

that function to provide molecular oxygen for oxidative metabolism to the ischemically damaged and injured organ. Such oxygen carrying agents are known to those skilled in the art to include, but are not limited to, hemoglobin, stabilized hemoglobin derivatives (made from hemolyzed human or bovine erythrocytes such as pyridoxylated hemoglobin), polyoxethylene conjugates (PHP), recombinant hemoglobin products, perfluorochemical (PFC) emulsions and/or perfluorochemical microbubbles (collectively referred to as "perfluorochemical"). Such oxygen carrying agents comprise from about 0% to about 50% by volume of the novel combination of supplements which are added to, and dissolved in, the base solution in forming the defatting solution of the present invention; or about 0% to about 20% of the total defatting solution (v/v).

In a process for preparing the defatting solution according to the present invention, to a base solution is added and dissolved therein a novel combination of supplements that can be grouped in at least 2 component categories comprising hormones and amino acids. Although the composition of the defatting solution, for use with the process according to the present invention, can vary by component and component ranges as previously described, a preferred formulation is set forth below in Table 1 for purposes of illustration and not limitation.

The defatting solution thus prepared has an osmolarity >280 mOsm but preferably less than 600 mOsm, and in a preferable range of about 300 mOsm to about 350 mOsm. The pH of the resuscitation solution is typically adjusted to a pH within a pH range of about 6.5 to about 7.8, and preferably in a pH range of 7.3 to 7.45. The defatting solution may also be heated to a temperature of 25 to 40^{0}C, but preferably, is heated to 34 to 39^{0}C. The solution may also be exposed to 20 to 100% O_2 and 0 to 10% CO_2, but preferably 95% O_2 and 5% CO_2.

In still another embodiment, the defatting solution may further include antioxidants, oxygen carrying agents, ions, carbohydrates, vitamins, agents that can provide oncotic pressure and pH indicators as indicated in Table 1.

TABLE 1. Composition of a perfusate solution for defatting livers.

Component	Concentration*
Salts and Carbohydrates	
Sodium chloride	116
Potassium sulfate	2.3
Sodium phosphate, monobasic	1.0
Sodium bicarbonate	26
Calcium chloride	1.9
Magnesium sulfate	0.81
Glucose	5.6
Amino Acids	
Alanine	0.48
Arginine	0.72
Asparagine	0.78
Aspartate	0.063
Cysteine	0.26
Glutamate	0.33
Glutamine	2.00
Glycine	0.38
Histidine	0.27
Isoleucine	0.40
Leucine	0.40
Lysine	0.50
Methionine	0.10
Phenylalanine	0.19
Proline	0.42
Serine	0.63
Threonine	0.40
Tryptophan	0.049
Tyrosine	0.29
Valine	0.39
Hormones	
Insulin	20 μU/mL
Glucagon	100 pg/mL
Epinephrine	250 pg/mL
Hydrocortisone	150 ng/mL
Anti-oxidants and Cytoprotective Agents	
N-acetyl-cysteine	2.0
Adenosine	5.0
Glutathione	3.0
Allopurinol	1.0
Vitamins and Others	
Hydroxyethyl starch	60.0 g/mL
Choline chloride	7.1×10^{-3}
Folic acid	2.3×10^{-3}
Myo-inositol	11×10^{-3}
Niacinamide	8.2×10^{-3}
Pantothenic acid	4.2×10^{-3}
Pyridoxal HCl	4.9×10^{-3}
Riboflavin	0.27×10^{-3}
Thiamine HCl	3.0×10^{-3}
Phenol red	31×10^{-3}

* All values are in mM except otherwise indicated.

Donor Transplant Material

A donor cell is obtained from virtually any source, autologous or heterologous, including kidney, heart, liver, lung, intestine, pancreas, bone marrow, and eye. Similarly, a donor tissue or organ includes, with out limitation, kidney, heart, liver, lung, intestine, pancreas, bone marrow, and eye.

Regimen/Apparatus/Timing

Cells, tissues, and organs can be defatted by simple incubation with the solution disclosed in Table 1. Any cell, tissue or organ in which reduction of intracellular lipid material is desirable, including, for example, the liver, the kidney, the pancreas, the heart, the lung, the small bowel, the brain, the eye, or the skin may be contacted or perfused with the defatting solutions disclosed herein.

If desired, cells, tissues, or organs are perfused with a defatting solution using the perfusion apparatus shown in Figure 1. As a specific example, the liver can be immersed in the perfusate solution and perfused via the portal vein at a rate of 4mL/min/g of liver. Perfusion rate can range between 1 mL/min/g to 5 mL/min/g, but preferably, perfusion should take place between 3 mL/min/g to 4 mL/min/g. The perfusate solution is heated to 37^0C through a heat exchanger and oxygenated by passing through a thin silicone tubular membrane exposed to 95% oxygen and 5% carbon dioxide. A bubble trap may be placed immediately before the perfusate enters the liver.

Cells, tissues, and organs can be treated with the defatting solution according to standard methods for a period of time sufficient to enable defatting, including, 10 minutes, 30 minutes, 1 hour, 2 hours, or more than 2 hours. In

preferred embodiments, a donor cell, tissue, or organ is treated with a deffating solution for two to three hours.

Assessment of Fat Content in Donor Cells, Tissues, or Organs

Fat content of donor cells, tissues, or organs is performed according to standard methods in the art. For example, ^{133}Xenon hepatic retention can be used as an accurate index for fatty liver quantification (Ahmad *et al., J. Nucl. Med.* 20: 397-401, 1979; Yeh *et al., J. Nucl. Med.* 30: 1708-1712, 1989). A less invasive method is based on the fact that the peak resonance frequency of ^{1}H nuclei of water differs significantly from that of aliphatic carbons (-CH_2-); proton chemical shift magnetic resonance imaging proved to be a sensitive and accurate way to evaluate the localization and quantity of fat deposits in liver and even bone marrow (Rosen *et al., Radiology* 169: 469-472, 1985; Rosen *et al., Radiology* 169: 799-804, 1988).

Storage/Preservation

The defatting solutions of the invention can be used to store, preserve and or protect cells, tissues, or organs, when these materials are brought into contact with the solution. A specific embodiment of the invention is for the preservation or storage of a human liver, or human liver tissue or cells. Another embodiment of the invention is for the preservation of a human heart or human heart tissue or cells. The invention contemplates the use of the defatting solutions to preserve mammalian cells, tissue, organs or portion thereof. In addition, the solutions can be used to facilitate transplantation of organs, e.g., by perfusion of the organ or tissue during the transplantation procedure. Preferably, the organ or portion

thereof, is maintained in the appropriate solution at all times.

The defatting solutions of the invention can be used to maintain viability of cells, tissues, or organs during storage, transplantation or other surgery. The invention includes a method of storing cells, tissues, or organs comprising contacting a donor cell, tissue, or organ, with the solution of the invention, such that the in vivo and/or in vitro viability is prolonged. The solutions permit maintenance of viability of a cell, tissue, or organ (e.g., a liver, heart, or lung) for up to 24 hours. Use of the solutions of the invention results in improved viability.

Kits

The present invention advantageously provides convenient kits for use by practitioners in the art for conveniently preparing a donor cell, tissue, or organ for transplantation into a recipient. In a preferred embodiment, a kit of the invention will provide sterile components suitable for easy use in the surgical environment. A kit of the invention may also provide sterile, defatting solution for preparing a donor cell, tissue, or organ for transplantation into a recipient. Generally, such a kit will include a defatting solution as described herein in appropriate containers, and optimally packaged with directions for use of the kit. For example, a kit of the invention can provide in an appropriate container or containers: (a) a predetermined amount of at least one defatting solution; (b) if necessary, other reagents; and (c) directions for use of said kit.

Transplantation

Once a cell, tissue, or organ is processed using the procedures described herein, such donor material is transplanted into a recipient (e.g., a human) according to standard methods known in the art.

The following experimental examples are provided for the purpose of illustrating the invention, and should not be construed as limiting.

Experimental

Modulation of Lipid Accumulation in Hepatocytes Cultured in Plasma

It has previously been shown that collagen-sandwiched adult rat hepatocytes which are seeded and maintained in standard hepatocyte culture medium and then exposed to either rat or human plasma become severely steatotic within 24 hours with a concomitant reduction in liver-specific functions (Matthew *et al., Biotechnol. Bioeng.* 51: 100-111, 1995; Stefanovich *et al., J. Surg. Res.* 66: 57-63, 1996). More recently, we found that intracellular accumulation of lipids occurs during exposure to plasma if hepatocytes are cultured in a medium containing high levels of insulin (e.g., similar to that found in standard hepatocyte culture medium or 500 mU/mL) prior to plasma exposure (Chan *et al., Biotechnol. Bioeng.* 78: 753-760, 2002). On the other hand, hepatocytes cultured in medium containing low insulin levels (50 μU/mL) exhibited little triglyceride accumulation during subsequent plasma exposure. In addition, triglyceride accumulation could be further reduced by direct plasma supplementation with an amino acid cocktail

as disclosed in Table 1 (Figure 2).

We also measured the expression of various liver-specific functions by hepatocytes exposed to plasma. We found that despite the tremendous accumulation of intracellular lipids, amino acid supplementation to the plasma allows hepatocytes to maintain the production of albumin and urea, as well as cytochrome P450 activities to levels similar to or even higher than hepatocytes in standard culture medium (Washizu *et al., J. Surg. Res.* 93: 237-246, 2000; Washizu *et al., Tissue Eng.* 6: 497-504, 2000; Washizu *et al., Tissue Eng.* 7:691-703, 2001). Thus, we concluded that by culturing hepatocytes in high insulin-containing hepatocyte culture medium followed by exposure to plasma supplemented with amino acids, we could obtain steatotic hepatocytes expressing high levels of liver-specific function. Recalling that steatotic livers do not generally show impaired functions in the absence of stressful conditions, these hepatocytes would appear to be a suitable model of steatotic liver.

In the next set of experiments, we induced steatosis in hepatocytes by exposing them to high insulin levels followed by plasma for 2 days, and attempted to defat them using the following conditions: plasma supplemented with amino acids and low insulin levels; culture medium containing high insulin (500 mU/mL); culture medium containing low insulin (50 μU/mL) levels. We then measured the fraction of remaining triglycerides after 1 and 2 days of treatment. Low insulin-containing medium almost completely removed intracellular triglycerides (Figure 3 and Table 2). The triglyceride removal kinetic data in Table 2 was used to calculate a defatting rate for each defatting condition. For this purpose, the fraction of initial triglyceride remaining was plotted as a function of time on a semi-log plot, which yielded linear curves (not shown). The slopes of

these lines, which correspond to the first order rate of decay or triglyceride clearance from the cells during defatting, are shown in Table 2 for each defatting condition tested. From these values, we can predict the fraction of intracellular lipid remaining after any treatment time using the simple equation:

$$\text{Triglyceride Fraction Remaining} = 100e^{-[\text{Rate Constant}][\text{Treatment Time}]} \quad \text{(equation 1)}$$

TABLE 2

"Defatting" Medium	Initial Triglyceride % Remaining*		1st Order Decay Rate Cst (h^{-1})	Metabolic Rates for 1st Day of Defatt ($\mu g/10^6$ cells/day)		
	Day 1	Day 2		Triglyceride Removal	Triglyceride Secretion	Keto Bod Secreti
Plasma, 50 μU/mL insulin + amino acids	85	62	0.010	108	-32	130
Medium, 500 mU/mL insulin	74	52	0.014	170	194	47
Medium, 50 μU/mL insulin	30	4	0.067	273	384	96

* Initial intracellular triglyceride content was 583±120 $\mu g/10^6$ cells.

Using the low insulin-containing medium, which was the most efficient at defatting, we can estimate that a treatment time of about 10 hours would be sufficient to remove 50% of the intracellular triglycerides, and 28 hours to remove 85%, the latter of which would correspond to normalizing the triglyceride content of a severely steatotic liver. This was a surprising result considering that a limited number of defatting conditions were tried, and it is expected that further optimization of this protocol will significantly reduce these defatting times. It is important to note that liver-specific functions, as determined by the albumin and urea secretion rates, were not reduced during exposure to this medium.

To determine the mechanism of defatting, we measured the triglyceride and ketone body secretion rates in the medium. We found that both of these rates were higher in the low insulin compared to the high insulin-containing medium. In hepatocytes continuously exposed to plasma, there was no net secretion of triglycerides, which probably explains the slower defatting rate. It is interesting to note that the total mass of triglycerides released into the medium exceeded the rate

of defatting, especially in the low insulin-containing medium, suggesting that a significant part of the triglycerides released arises from de novo synthesis in hepatocytes. Thus, it is anticipated that addition of drugs which inhibit triglyceride or free fatty acid synthesis (e.g. see Loftus *et al.*, *Science* 288: 2379-2381, 2000) could significantly accelerate the rate of triglyceride clearance from hepatocytes. We also investigated the effects of leptin and hepatocyte growth factor on the defatting process. In low insulin-containing medium, these agents did not further enhance lipid removal. Some fat-reducing effects were seen in high insulin containing media, albeit not as dramatic as the reduction observed by lowering the insulin concentration.

Response of Steatotic Hepatocytes to Ischemia/Reperfusion

To investigate whether I/R injury correlates with the level of triglyceride loading in hepatocytes, we studied the response of normal and steatotic hepatocytes to I/R. Steatotic hepatocytes were generated by exposure to plasma supplemented with 500 mU/mL insulin and amino acids for 2 days. I/R was induced by switching the cells to an atmosphere containing 90% N_2 and 10% CO_2 for various lengths of time, after which the cells were returned to normoxic conditions. Culture supernatants were harvested 12 hours after restoration of normoxia for the determination of lactate dehydrogenase release, a measure of cell lysis. Lactate dehydrogenase activity in the supernatant was normalized to that of dead controls (hepatocytes subjected to rapid freeze-thaw). We found that steatotic hepatocytes are more sensitive to I/R than lean hepatocytes (Figure 4, left panel). To determine whether the lipid content at the time of I/R is what determines the sensitivity of cells to I/R, hepatocytes were defatted for different

lengths of time prior to I/R. Cell lysis after I/R decreased as a function of defatting time (Figure 4, right panel).

In order to provide additional evidence that the lipid load indeed determines the resistance of cultured hepatocytes to I/R, we investigated the effect of cold storage followed by rewarming on hepatocyte lysis. Hepatocytes were made steatotic by culturing in plasma for 2 days, after which they were incubated in the UW solution at 4^0C for 12 hours. The cells were then returned to standard hepatocyte culture medium at 37^0C for 12 hours, and the release of lactate dehydrogenase in the medium was determined. Consistent with prior observations, lactate dehydrogenase release correlated with the amount of intracellular lipids in the hepatocytes (Figure 5). As a preliminary assessment of the potential mechanisms of death in this cell culture model, we measured cytochrome c release from the mitochondrial to the cytosolic fraction of the cells, an indicator of apoptosis. Cytochrome c was quantified on Western blots of cytosolic and mitochondrial fractions of hepatocytes subjected to different defatting regimen leading to varying triglyceride content at the time of I/R. We found that cytochrome c release was significantly correlated ($p<0.006$) with triglyceride storage in hepatocytes (Figure 6), consistent with the greater extent of cell death shown in Figure 5.

Effect of Liver Nonparenchymal Cells on the Hepatocyte Response to I/R in Co-cultures

Since various in vivo studies suggest that Kupffer cells may be activated by I/R and exacerbate the injury (Lichtman and Lermasters, *Sem. Liver Dis.* 19: 171-187, 1999), we investigated the effect of nonparenchymal cells on the response of

steatotic hepatocytes to I/R in micropatterned co-cultures. Hepatocytes were patterned as islands of sizes ranging from 36 to 490 μm on tissue culture dishes using stencil technology described by Folch *et al.* (*J. Biomed. Mater Res.* 52: 346-353, 2000; *Ann. Rev. Biomed. Eng.* 2: 227-256, 2000). The nonparenchymal cell fraction obtained from another liver cell isolation was then seeded on top of the hepatocytes. Nonparenchymal cells only attach to the vacant spaces left in-between the hepatocyte islands. Thus, one can increase direct hepatocyte-nonparenchymal cell interactions by reducing the size of hepatocyte islands, and vice-versa (Bhatia *et al., J. Biomed. Mat. Res.* 34: 189-199, 1997; Bhatia *et al., Biotechnol. Prog.* 14: 378-387,1998; Bhatia *et al., J. Biomater. Sci. Polym. Ed.* 9:1137-1160, 1998). The cultures were then exposed to plasma supplemented with high insulin levels and amino acids for 2 days to cause steatosis. Five minutes before starting the I/R experiment, 1 μM calcein acetoxymethyl ester was added to the cells for 5 minutes. This compound is specifically retained and converted to brightly fluorescent calcein within viable cells and released upon membrane rupture at the time of cell death.

A small flow device made by micro-molding of polydimethylsiloxane as described elsewhere (Folch and Toner, *Biotechnol. Prog.* 14: 388-392, 1998) was placed on top of the cells to create a mini cell perfusion bioreactor. The bioreactor was perfused with medium saturated with 90% air/10% CO_2 for 1 hour. The flow was then stopped for 1 hour. Because of the low aspect ratio of the flow channel above the cells (1 cm long, 1 cm wide, and 100 μm high), hypoxia occurs inside the flow channel within a few minutes, which mimics the situation in the actual liver when blood flow is stopped. Flow was then restored and cells visualized for an additional 5 hours. The I/R experiment was set up on the temperature-

controlled stage of an inverted fluorescence microscope fitted with a digital video camera and image analysis software to quantify the fluorescence intensity distribution of at regular times intervals. Since in these experiments we were primarily interested in hepatocyte viability, the intensity of calcein fluorescence per surface area over hepatocyte islands only was measured, averaged for each island size, and normalized to that measured initially. Hepatocyte viability, based on the fraction of initial calcein fluorescence intensity, decreased as a function of time after reoxygenation and was lower in the smaller hepatocyte islands (Figure 7). In addition, hepatocyte viability decreased as a function of hepatocyte island size in co-cultures and was lower in co-cultures than in pure hepatocyte cultures. These data strongly support the hypothesis that nonparenchymal cells have deleterious effects on hepatocyte viability after I/R. The data also show that steatotic hepatocytes are more sensitive to I/R than lean hepatocytes, confirming our earlier data based on lactate dehydrogenase release in static cultures.

Non-Invasive Imaging of Hepatic Lipid Metabolism

Non-invasive quantitation of hepatic lipid content and metabolism is potentially very useful to optimize and monitor the effect of defatting regimens. In prior studies, we have shown that proton chemical shift nuclear magnetic resonance (NMR) imaging can provide a quantitative measurement of the liver fat content (Rosen *et al., Radiology*, 154: 469-472, 1985). In these experiments, rats were either alcohol-fed or received an intraperitoneal injection of ethionine, a protein synthesis inhibitor, to cause lipid accumulation. The NMR signal intensity was directly proportional to the hepatic triglyceride content measured using a biochemical assay (Figure 9).

This technique is noninvasive and does not require the animal or patient to undergo any particular preparatory procedures, except for the requirement of immobilization, as the imaging time takes about 45 minutes. More recently, we applied the same technique to non-invasively determine fat distribution in bone marrow of human patients (Rosen *et al., Radiology,* 169: 799-804, 1988). Later on, we found that the distribution of fat determined by this technique is a useful surrogate marker to monitor the severity of Gaucher disease and the efficacy of treatments against acute leukemia (Gerard *et al., Radiology,* 183:39-46, 1992; Johnson *et al., Radiology* 182:451- 455,1992). This technique may, if desired, be combined with other techniques to determine microvascular flow distribution and ATP levels in tissue during liver perfusions.

We have also developed methods to determine metabolic fluxes through the tricarboxylic acid and gluconeogenic pathways using ^{13}C-NMR spectroscopy and gas chromatography-mass spectroscopy, which we used to investigate metabolic changes in burned rats and patients (Vogt *et al., Am. J. Physiol.* 266:E1012-1022, 1994; Vogt *et al., Am. J. Physiol.* 272: C2049-2062, 1997; Yarmush *et al., J. Burn Care Rehabil* 20: 292-302, 1999). As part of these studies, we recently improved the mathematical formalism used to determine fluxes from ^{13}C isotopic distributions by implementing "atom mapping matrices," which allow one to quickly optimize labeling strategies and adapt the quantitative model for data analysis (Zupke *et al., Anal. Biochem.* 247: 287-293, 1997). This technology is useful in analyzing metabolic pathways of fatty acid oxidation and metabolism, and independently verify metabolic fluxes obtained with the stoichiometric mass balance model.

Because there are currently no real-time imaging techniques using NMR to

study carbon metabolism, we have used positron emission tomography (PET) to non-invasively monitor regional metabolism in burned patients. For example, previous studies in our laboratory have demonstrated that PET and parallel arterial sampling after bolus injection of L-[methyl-^{11}C]methionine and 1-[^{11}C]-3-R,S-methylheptadecanoic acid can provide less invasive, regional assessments of the protein synthetic rate and fatty acid oxidation rate, respectively, than traditional approaches (Zaknun, *J. Nucl. Med.* 36: 2062-2068, 1995; Hsu *et al., Proc. Natl. Acad. Sci. U.S.A.* 93: 1841-1846, 1996). In sum, we have established that PET can be used to study carbon metabolism in healthy human subjects and animals, and that it holds promise for future in vivo, non-invasive studies of the influences of physiological factors and pharmacological manipulations on regional metabolism (Fischman *et al., Proc. Natl. Acad. Sci. U.S.A.* 27: 12793-12798, 1998).

Defatting of Rat Livers Restores Survival of Recipients

To induce hepatic steatosis in rats, rats (age/weight), prior to surgery, were fed a choline- and methionine-deficient diet (CMDD) for 6 weeks as described by Nakano *et al.* (*Hepatology,* vol. 26, pp. 670-678, 1997). Rats fed a CMDD exhibited a time-dependent increase in liver triglyceride (TG) content from ~10 to 250 mg TG/g liver after 5-6 wk. This accumulation was reversible, as returning the animal back to a regular diet caused the hepatic TG content to return to normal (Figure 9).

CMDD fed rats were returned to a regular diet for 3 or 7 days before harvesting the livers for transplantation. The donor livers were removed and stored as follows. After laparotomy, the bile duct of the liver was cannulated with a short polyethylene tube. Veins emptying into the portal vein and the hepatic

artery were subsequently ligated and divided, and the portal vein was divided at the level of the inferior mesenteric vein. To prepare the portal vein cuff, a short polyethylene tube was slipped over the vein and the vein everted over the tube. The infrahepatic vena cava and suprehepatic vena cava, including part of the diaphragm, were then transected. The liver is flushed with hetastarch-free UW solution and stored in a reservoir containing the same for 6 hours at 0^0C.

The donors were stored in a hetastarch-free UW preservation solution for 6 hours at 4^0C, and then transplanted into a recipient rat as follows. The recipient animal was prepared by cannulating the bile duct, clamping the portal vein, and tying shut the other vessels. The liver was removed and discarded. The donor liver is placed orthotopically, the suprahepatic vena cava anastomosed, and the cuffed portal vein was inserted into the recipient's portal vein. Blood is then allowed to flow into the donor liver, and the infrahepatic vena cava is anastomosed. The bile duct is reconnected and wrapped around the omentum. The abdominal incision is then closed. This protocol mimics the clinical situation which typically requires that the liver be preserved in the UW solution for several hours while it is being transported from the donor to the recipient site.

As is shown in Figure 9, the control animals receiving untreated fatty livers show no survival after 4 days. In contrast, recipients of defatted livers show a complete recovery of survival rate, with no statistically significant difference in survival when compared to recipients receiving control (nonfatty) livers.

Metabolic Conditioning of Steatotic Perfused Livers to Reduce Their Lipid Content

Based on our cell culture data, we tested the effect of warm perfusion with

buffer containing no insulin and high glucagon (10 ng/mL) on the triglyceride content of steatotic livers. Donor livers were prepared for transplantation and then perfused at 37^{0}C as follows. Steatotic livers were obtained by feeding rats a CMDD for 6-7 weeks. The buffer also contained 3% bovine serum albumin in order to prevent tissue swelling. Perfusions were carried out at a flow rate of 4 mL/min/g liver, 37^{0}C, and using 95% O_2/5% CO_2 for 1-3 hours in a recirculating mode. The perfusate solution consists of Minimal Essential Medium supplemented with hydroxyethyl starch (6% w/v), amino acids, glucagon, hydrocortisone, and anti-oxidants.

The triglyceride content of livers was measured after the perfusion and compared to that of unperfused livers from rat littermates. Initially, we compared buffer vs. amino acid-containing medium, and found a significantly increased rate of triglyceride clearance in the presence of amino acids (Figure 10A). Using amino acid-supplemented medium, we investigated the kinetics of clearance during the first 3 hours of perfusion, and found a linear relationship (Figure 10B). After 3 hours, warm perfusion reduced the triglyceride content of fatty livers by 85%. These data demonstrate that warm perfusion can be used to reduce the hepatic lipid storage of fatty livers.

In addition, as is shown in Figure 10, the TG content decreased as a function of time and the defatting process was largely complete after 3 hours. It is likely that there are two major mechanisms of action of the defatting regimen. First, the catabolic hormones glucagon and hydrocortisone, which are in the perfusate, favor the oxidation of lipids, more specifically fatty acids. Second, the amino acids in the perfusate provide the building blocks required for the synthesis of apolipoproteins, which are then incorporated into the larger lipoproteins. These

lipoproteins export TG and other lipids (e.g. cholesterol) outside of the cell.

It is interesting to note that, based solely on typical measured oxygen uptake rates of perfused livers, one would predict a maximum possible rate of lipid oxidation about one order of magnitude less than observed in Figure 9, suggesting that other pathways of defatting (e.g. export of triglycerides in the form of lipoproteins) are probably very important in this process. In addition, using the triglyceride clearance equation (equation 1) fitted to cell culture data earlier would predict a decrease to 82% of the original lipid load after 3 hours of treatment with low insulin medium (as compared to the 85% measured), suggesting that our steatotic hepatocyte culture model closely reflects the behavior of fatty livers, and thus can be used to rapidly screen for more effective defatting regimens.

To summarize, fatty livers are very sensitive to ischemia-reperfusion and cold preservation-related injuries, which makes them unacceptable for liver transplantation. We hypothesized that removal of the excess fat storage from fatty livers can restore their ability to undergo liver transplantation. We obtained fatty livers from rats fed a CMDD for 6 wk, stored them in cold hetastarch-free UW solution for 6 hours, and transplanted them into normal recipient rats. While recipient rats had a 90% rate of survival after transplantation of control normal lean livers, they all died when receiving CMDD rat livers. If CMDD rats were returned to a normal diet for 3 or 7 days prior to donating livers, effectively reducing the fat content of the livers by 33% and 85%, respectively, the recipients survived at rates similar to the controls. Furthermore, we found that it is possible to eliminate excess fat storage from fatty livers by short-term perfusion of fatty livers ex vivo. These results support the notion that liver perfusion could be used to recondition fatty livers and make them suitable for transplantation.

Other Embodiments

Other embodiments are within the claims.

All publications mentioned in this specification are hereby incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

Claims

1. A method for preparing a donor cell, tissue, or organ for transplantation into a recipient, said method comprising reducing intracellular lipid storage material of said cell, tissue, or organ.

2. The method of claim 1, wherein said cell is prepared.

3. The method of claim 1, wherein said tissue is prepared.

4. The method of claim 1, wherein said organ is prepared.

5. The method of claim 1, wherein said cell is a liver cell.

6. The method of claim 1, wherein said tissue is liver tissue.

7. The method of claim 1, wherein said organ is a liver.

8. The method of claim 1, wherein said method comprises contacting said cell, tissue, or organ with a solution that increases oxidation of a lipid.

9. The method of claim 1, wherein said method comprises contacting said cell, tissue, or organ with a solution that increases export of a lipid from said cell, tissue, or organ.

10. The method of claim 1, wherein said method comprises contacting said cell, tissue, or organ with a solution that increases oxidation and export of a lipid from said cell, tissue, or organ.

11. The method of claim 1, wherein said intracellular lipid storage material is a triglyceride, a cholesterol, a cholesterol ester, or a phospholipid.

12. The method of claim 1, wherein said method results in reducing an ischemia-reperfusion injury in said cell, tissue, or organ upon transplantation into a recipient.

13. The method of claim 1, wherein said method results in reducing a cold-preservation-related injury in said cell, tissue, or organ upon transplantation into a recipient.

14. The method of claim 1, wherein said method reconditions a steatotic cell, tissue, or organ.

15. The method of claim 14, wherein said steatotic cell is a liver cell.

16. The method of claim 14, wherein said steatotic tissue is liver tissue.

17. The method of claim 14, wherein said steatotic organ is a liver.

18. A solution for reducing intracellular lipid storage material of a

donor cell, tissue, or organ for transplantation into a recipient, comprising a catabolic hormone and an amino acid.

19. The solution of claim 18, wherein said catabolic hormone increases intracellular lipid oxidation.

20. The solution of claim 18, wherein said catabolic hormone increases lipid export.

21. The solution of claim 18, wherein said catabolic hormone is glucagon, epinephrine, growth hormone, hepatocyte growth factor, leptin, thyroid hormone, or a glucocorticoid hormone.

22. The solution of claim 21, wherein said glucocorticoid hormone is a hydrocortisone, a cortisol, a corticosterone, or dexamethasone.

23. The solution of claim 18, wherein said amino acid is required for the synthesis of an apolipoprotein.

24. The solution of claim 23, wherein said amino acid is alanine or glutamine.

25. The solution of claim 18, wherein said solution further comprises an anti-oxidant.

26. The solution of claim 25, wherein said anti-oxidant is N-acetyl-cysteine, glutathione, allopurinol, or adenosine.

27. The solution of claim 18, wherein said solution further comprises an oxygen carrier.

28. The solution of claim 18, wherein said oxygen carrier is hemoglobin or a perfluorocarbon.

29. The solution of claim 18, said solution further comprising a component that provides oncotic pressure.

30. The solution of claim 18, comprising

from 50 mM to 150 mM sodium ion;

from 0.4 mM to 4 mM potassium ion;

from 0 mM to 50 mM phosphate ion;

from 0 mM to 44 mM bicarbonate ion;

from 0.19 mM to 5 mM calcium ion;

from 0.081 mM to 5 mM magnesium ion;

from 0.2 mM to 2.4 mM alanine;

from 0.2 mM to 10 mM glutamine;

from 50 pg/mL to 1000 pg/mL glucagon;

from 100 pg/mL to 2500 pg/mL epinephrine;

from 50 ng/mL to 1500 ng/mL hydrocortisone; and

from 30 g/mL to 120 g/mL hydroxyethyl starch.

31. The solution of claim 30, comprising

116 mM sodium ion;

2.3 mM potassium ion;

1.0 mM sodium phosphate (monobasic);

26 mM sodium bicarbonate;

1.9 mM calcium ion;

0.81 mM magnesium ion;

0.48 mM alanine;

2.00 mM glutamine;

100 pg/mL glucagon;

250 pg/mL epinephrine;

150 ng/mL hydrocortisone; and

60.0 g/mL hydroxyethyl starch.

32. The solution of claim 18, wherein said cell is a liver cell.

33. The solution of claim 18, wherein said tissue is liver tissue.

34. The solution of claim 18, wherein said organ is a liver.

35. The solution of claim 18, wherein said solution is heated to a temperature of 25 to 40 ^{0}C.

36. The solution of claim 35, wherein said solution is heated to 37^0C.

37. The solution of claim 18, wherein said solution is exposed to 20 to 100 % O_2.

38. The solution of claim 37, wherein said solution is exposed to 95% O_2.

39. The solution of claim 18, wherein said solution is exposed to 0 to 10% CO_2.

40. The solution of claim 39, wherein said solution is exposed to 5% CO_2.

41. The solution of claim 18, wherein said solution has a pH of 6.5 to 7.8.

41. The solution of claim 41, wherein said solution has a pH of 7.4.

43. A method for preparing a donor cell, tissue, or organ for transplantation into a recipient, said method comprising contacting said donor cell, tissue, or organ with the solution of claim 18, 30, or 31.

44. The method of claim 43, wherein said solution is heated to a temperature of 37^0C.

45. The method of claim 43, wherein said solution is exposed to 95% O_2 and 5% CO_2.

46. The method of claim 43, said method comprising contacting said cell, tissue, or organ for at least one hour with said solution.

47. The method of claim 43, wherein said solution has a pH of 7.4.

48. A method for reconditioning a steatotic cell, tissue, or organ for transplantation into a recipient, said method comprising contacting said cell, tissue, or organ with the solution of claim 18, 30, or 31.

49. The method of claim 48, wherein said solution is heated to a temperature of 37^0C.

50. The method of claim 48, wherein said solution is exposed to 95% O_2 and 5% CO_2.

51. The method of claim 48, said method comprising contacting said cell, tissue, or organ for at least one hour with said solution.

52. The method of claim 48, wherein said solution has a pH of 7.4.

53. A method of storing a donor cell, tissue, or organ for transplantation

into a recipient, said method comprising contacting said donor cell, tissue, or organ with the solution of claim 18, 30, or 31.

54. The method of claim 53, wherein said solution is heated to a temperature of 37^0C.

55. The method of claim 53, wherein said solution is exposed to 95% O_2 and 5% CO_2.

56. The method of claim 53, said method comprising contacting said cell, tissue, or organ for at least one hour with said solution.

57. A kit for preparing a donor cell, tissue, or organ for transplantation into a recipient, said kit comprising a solution for reducing intracellular lipid storage material of said donor cell, tissue, or organ and instructions for using said solution for preparing a donor cell, tissue, or organ for transplantation into a recipient.

58. The kit of claim 57, said kit comprising the solution of claim 18, 30 or 31.

59. A kit for reconditioning a steatotic cell, tissue, or organ for transplantation into a recipient, said kit comprising a solution for reducing intracellular lipid storage of said steatotic cell, tissue, or organ and instructions for using said solution for reconditioning said steatotic cell, tissue, or organ for

transplantation into a recipient.

60. The kit of claim 59, said kit comprising the solution of claim 18, 30, or 31.

61. A kit for storing a donor cell, tissue, or organ for transplantation into a recipient, said kit comprising a solution for reducing intracellular lipid storage material in said donor cell, tissue, or organ and instructions for using said solution for storing a donor cell, tissue, or organ for transplantation into a recipient.

62. The kit of claim 61, said kit comprising the solution of claim 18, 30, or 31.

63. A cell, tissue, or organ prepared according to the method of claim 1, 43, 48, or 53.

64. An isolated defatted donor cell, tissue, or organ.

65. The isolated cell, tissue or organ of claim 64, wherein said cell, tissue, or organ is prepared for transplantation into a recipient.

66. The isolated cell, tissue or organ of claim 65, wherein a defatted cell is prepared.

67. The isolated cell, tissue or organ of claim 65, wherein a defatted tissue is prepared.

68. The isolated cell, tissue or organ of claim 65, wherein a defatted organ is prepared.

COMPOSITIONS, SOLUTIONS AND METHODS USED FOR TRANSPLANTATION

Abstract of the Disclosure

This invention discloses a method for reducing the intracellular lipid storage material of a cell, tissue, or organ for transplantation and features solutions, methods and kits that induce the metabolic elimination of lipid storage in a cell, tissue, or organ. The process comprises contacting a cell, tissue, or organ with a perfusate solution comprising catabolic hormones and amino acids, at physiological conditions, to increase lipid export and lipid oxidation. The invention can be used to prepare, recondition or store a cell, tissue, or organ for transplantation by increasing tolerance to ischemia-reperfusion and cold-preservation related injury.

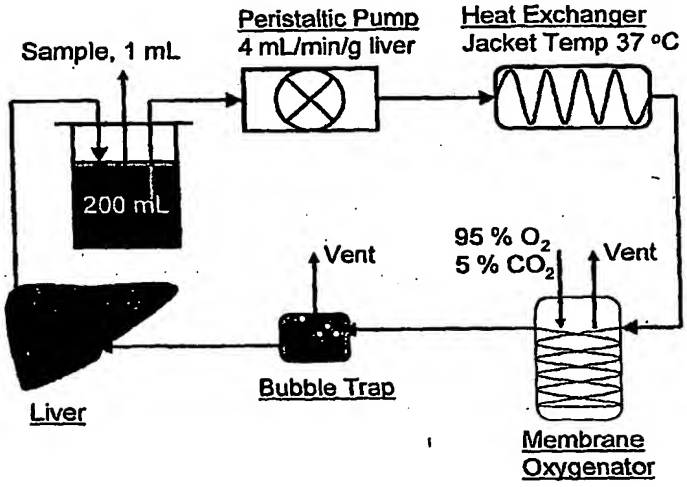
Peristaltic Pump
4 mL/min/g liver
Heat Exchanger
Jacket Temp 37 °C
Sample, 1 mL
200 mL
Vent
95 % O_2
5 % CO_2
Vent
Liver
Bubble Trap
Membrane
Oxygenator

FIGURE 1

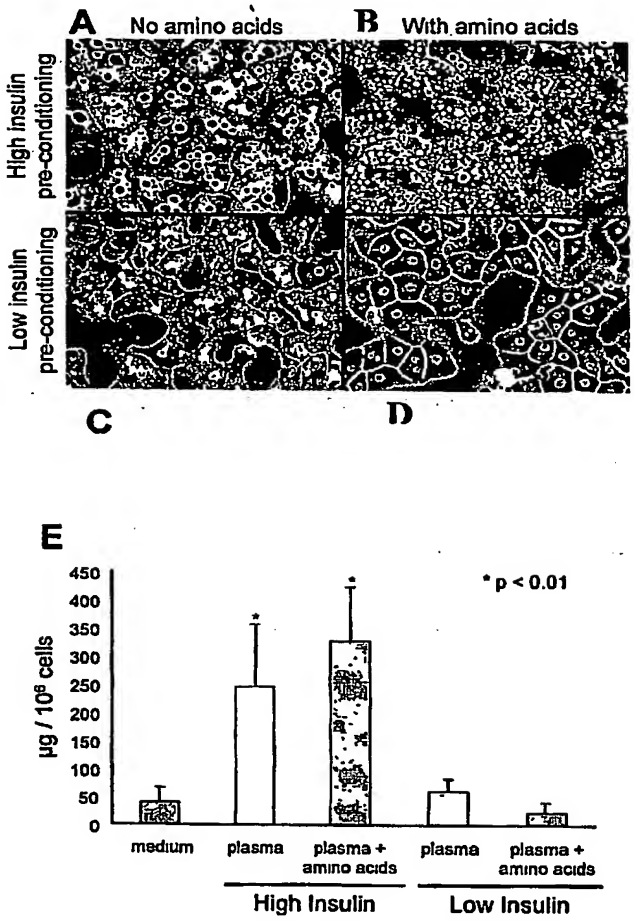
A
No amino acids
B
With amino acids
High insulin pre-conditioning
Low insulin pre-conditioning
C
D
E
* p < 0.01
µg / 10⁶ cells
450
400
350
300
250
200
150
100
50
0
medium
plasma
plasma + amino acids
plasma
plasma + amino acids
High Insulin
Low Insulin

FIGURE 2

Plasma
+ 50 µU/mL Insulin
+ Amino Acids

Medium
+ 500 mU/mL Insulin

Medium
+ 50 µU/mL Insulin

FIGURE 3

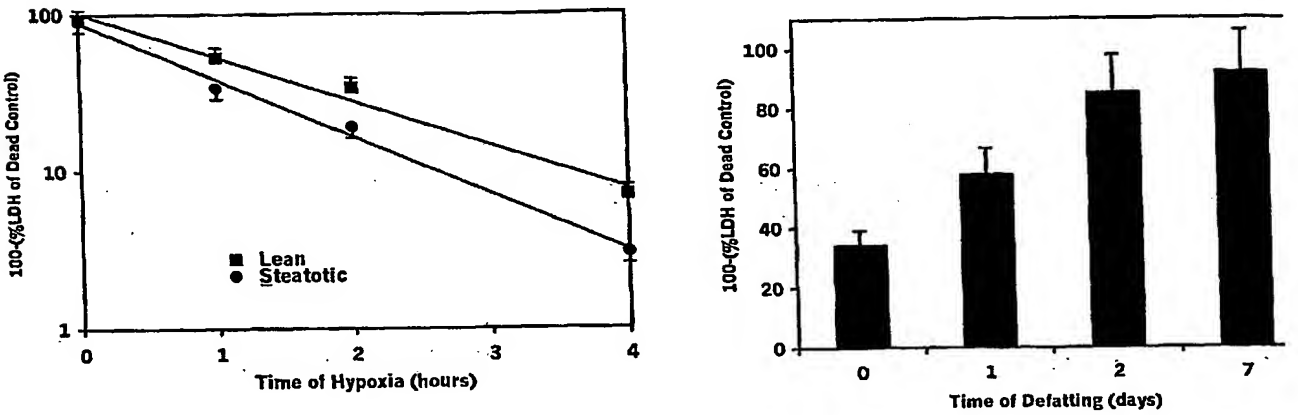
100
10
1
100-(%LDH of Dead Control)
Lean
Steatotic
0
1
2
3
4
Time of Hypoxia (hours)
100
80
60
40
20
0
100-(%LDH of Dead Control)
0
1
2
7
Time of Defatting (days)

FIGURE 4

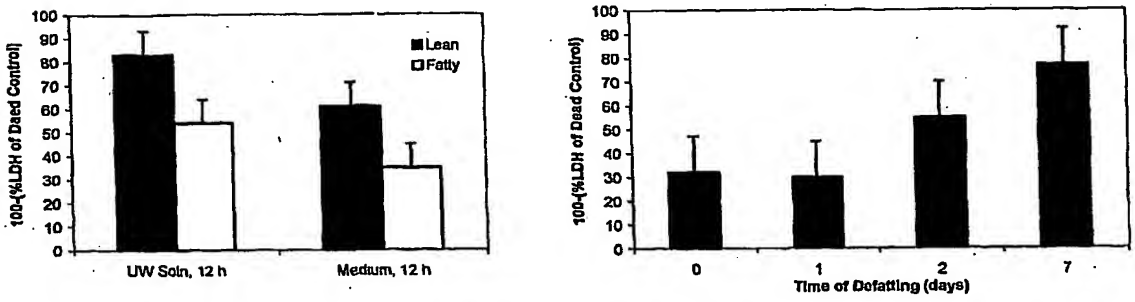
100-(%LDH of Daed Control)
Lean
Fatty
UW Soln, 12 h
Medium, 12 h
100-(%LDH of Dead Control)
Time of Defatting (days)

FIGURE 5

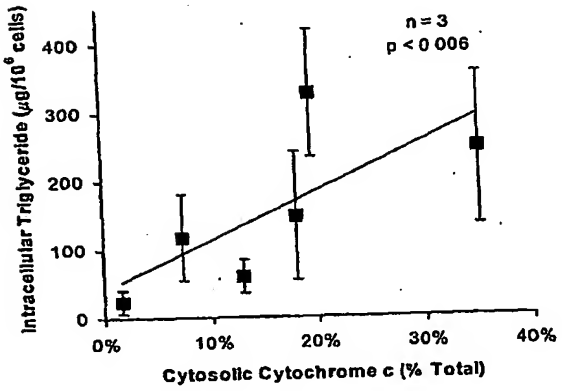
n = 3
p < 0.006
Intracellular Triglyceride (µg/10^6 cells)
400
300
200
100
0
0%
10%
20%
30%
40%
Cytosolic Cytochrome c (% Total)

FIGURE 6

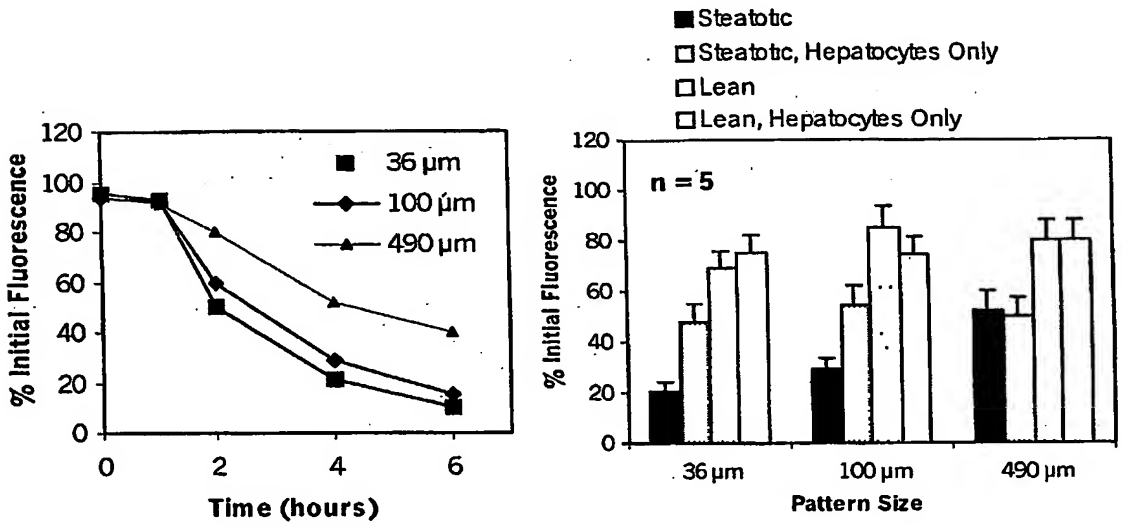
Steatotic
Steatotic, Hepatocytes Only
Lean
Lean, Hepatocytes Only
% Initial Fluorescence
120
100
80
60
40
20
0
36 µm
100 µm
490 µm
0
2
4
6
Time (hours)
n = 5
36 µm
100 µm
490 µm
Pattern Size

FIGURE 7

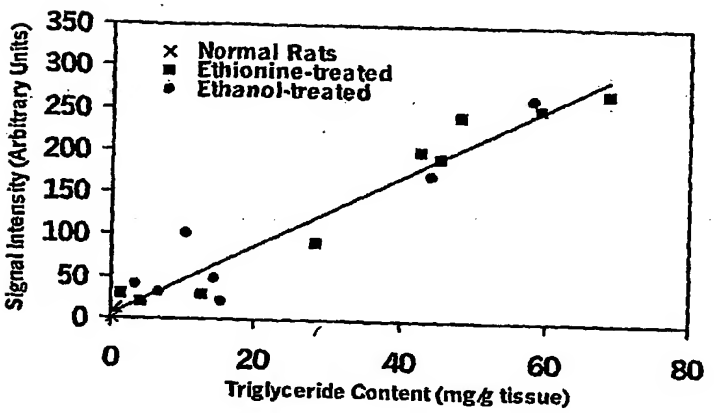
Signal Intensity (Arbitrary Units)
350
300
250
200
150
100
50
0
Normal Rats
Ethionine-treated
Ethanol-treated
0
20
40
60
80
Triglyceride Content (mg/g tissue)

FIGURE 8

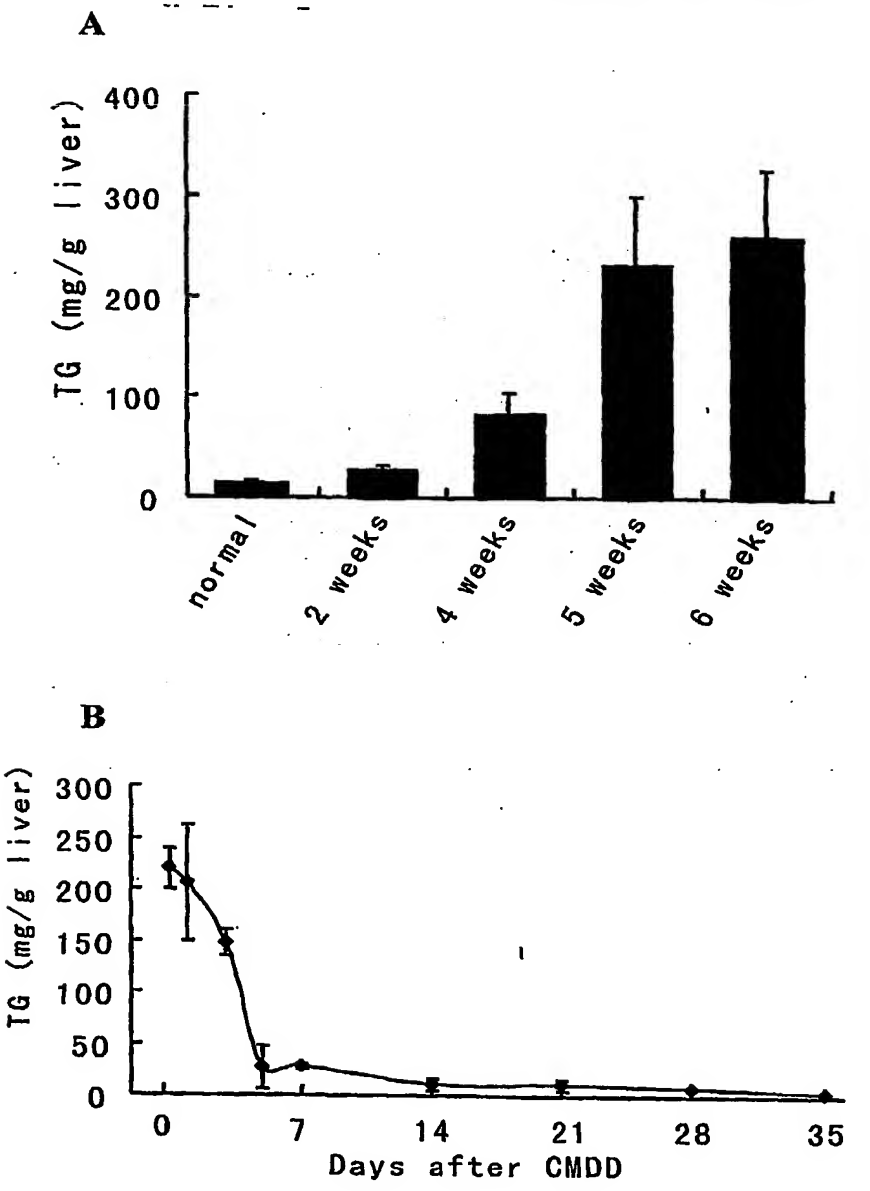
A
TG (mg/g liver)
400
300
200
100
0
normal
2 weeks
4 weeks
5 weeks
6 weeks
B
TG (mg/g liver)
300
250
200
150
100
50
0
0
7
14
21
28
35
Days after CMDD

FIGURE 9

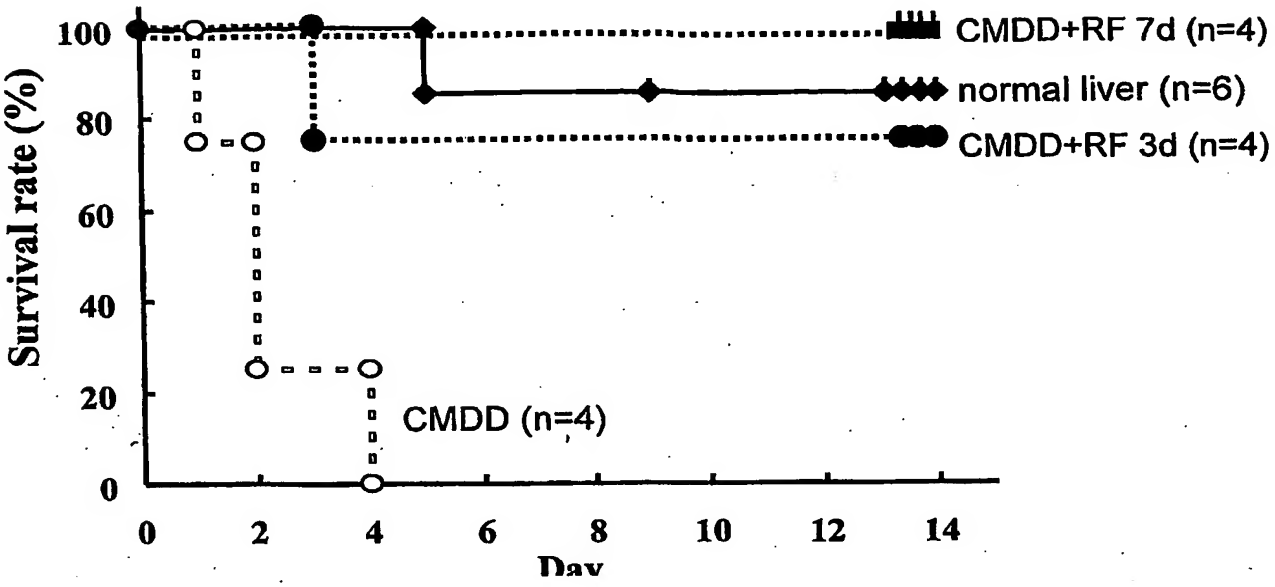
100
80
60
40
20
0
Survival rate (%)
0
2
4
6
8
10
12
14
Day
CMDD+RF 7d (n=4)
normal liver (n=6)
CMDD+RF 3d (n=4)
CMDD (n=4)

FIGURE 10

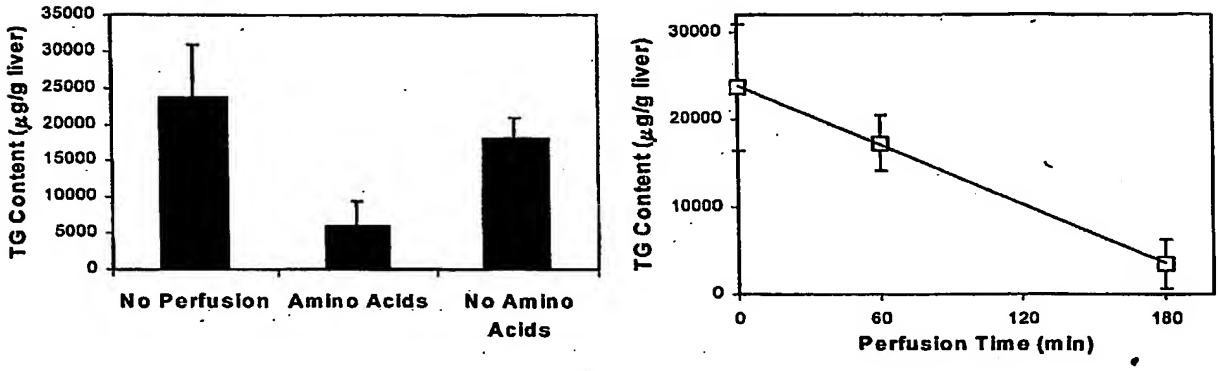
TG Content (µg/g liver)
35000
30000
25000
20000
15000
10000
5000
0
No Perfusion
Amino Acids
No Amino Acids
TG Content (µg/g liver)
30000
20000
10000
0
0
60
120
180
Perfusion Time (min)

FIGURE 11